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On the basis of an extensive body of cytogenetic literature as well as molecular evidence from our laboratory, we hypothesize that a tumor suppressor gene is located on the long arm of Human Chromosome 7 at the q31.1 band. Furthermore, we propose that inactivation of this tumor suppressor gene plays a role in the development of breast cancer.

The long term objectives of the study are to identify and clone the tumor suppressor gene and to determine its function. The objectives for the requested funding period are to provide functional evidence for the existence of this tumor suppressor gene in breast cancer using microcell fusion and to clone the DNA fragment containing the putative tumor suppressor gene using a novel approach based on the introduction of Yeast Artificial Chromosomes (YACs) into breast cancer cells. This novel approach will facilitate the cloning of putative tumor suppressor genes closing the technical gaps that exist between the identification of deleted regions of genome containing putative tumor suppressor genes and the actual cloning of DNA fragments containing the candidate genes.

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INTRODUCTION

Several reports in the literature indicate that karyotypic alterations of chromosome 7 are common in many different types of neoplasia including breast cancer (reviewed in Atkin and Baker, 1991; Pandis, et al. 1993). These alterations include monosomies, trisomies and deletions of different segments of the chromosome. More recently, several laboratories including our own have shown a high frequency of loss of heterozygocity (LOH) in the long arm of chromosome 7 suggesting the existence of a tumor suppressor gene in this region (reviewed in Zenklusen and Conti, 1996). Furthermore, microcell fusion experiments have shown that chromosome 7 can inhibit tumorigenicity or induce senescence in several cell lines (Zenklusen, et al. 1994b; Ogata, et al. 1993). On the basis of this cytogenetic, molecular and functional evidence, we hypothesize that a tumor suppressor gene is located in the long arm of chromosome 7 at the q31.1 band. Furthermore, we propose that inactivation of this tumor suppressor gene plays a role in the development of breast cancer.

The overall objective of these studies is to identify and/or clone this putative tumor suppressor gene using innovative approaches. Thus, we propose to do a number of experiments to develop this approach and to apply them to the cloning of this gene.

The proposed strategy consists of transferring the whole chromosome 7 as well as relevant fragment of this chromosome (as shown by our LOH studies) into breast cancer cell lines. Whole chromosomes can be transferred to the breast cancer cells by microcell fusion as described in previous experiments from our laboratory (Zenklusen, et al. 1994a). To transfer relevant fragments of chromosome 7 we propose to use spheroblast fusion with selected yeast artificial chromosomes (YACs) which contain the markers with higher LOH in breast cancer (Huxley and Gnirke, 1991; Markie, et al. 1993).

In the past period of the grant we performed microcell fusion with MCF-7 cells obtaining slow growth cultures and abortive colonies. These results are similar to the ones obtained in Carl Barret's laboratory (Ogata, et al. 1993) but differ from our previous results in rodent epithelial cells in which introduction of a chromosome 7 suppressed tumorigenicity with minimal effect on the in vitro proliferative potential (Zenklusen, et al. 1994a). Based on these results as well as data from our laboratory indicating that chromosome 7 can inhibit tumorigenicity in prostate cancer cells without affecting growth in tissue culture (Zenklusen, et al. manuscript in preparation), we postulate that two potential tumor suppressor genes may be present in chromosome 7, one affecting senescence and the other affecting the relationship between the tumor cells and the host. To further explore this possibility we have carried out LOH studies in a number of well characterized breast cell lines using highly polymorphic microsatellite markers to determine the pattern of chromosome alterations in these cell lines and to choose other lines for future studies.

During this period we have also performed YAC/spheroblasts fusions. However these experiments were also carried out on MCF-7 cells which as described above are not an adequate recipient cells for these studies. Therefore, these studies will be repeated

using a different cell lines. In addition, upon recommendation of our collaborators at the laboratory of E. Green (National Center of Genome Research), we incorporated the use of Bacterial Artificial Chromosomes (BACs) to replace the YACs to manipulate small fragments of chromosome 7. There is a strong rationale for this change. The BACs are more stable, do not produce chimeric recombination and the detailed physical map of chromosome 7 from Green's laboratory is based on BACs.

BODY

A. Experimental Procedures

Microcell fusion experiments: Chromosome 7 will be introduced in to the MCF-7 (breast cancer derived) (Brooks, et al. 1973) cells in order to determine whether this putative tumor suppressor gene is functional in human breast carcinoma cells as it is in murine squamous cell carcinoma (SCC) cells.

Microcell fusions: Microcell fusion experiments were performed as previously described by our laboratory using A9 cells containing a neo-tagged chromosome 7 as the donor cell line and MCF-7 as the recipient cell line (Zenklusen, et al. 1994a).

Spheroblast fusions: Selected YACs were transferred into the MCF cell line using the protocol described by Markie, et al. (1993). Briefly, the yeast containing the selected YACs were grown overnight at 30°C in 5ml SD medium (0.17% yeast nitrogen base w/o amino acids and ammonium sulfate, 0.50% ammonium sulfate, 2% dextrose and pH 5.8) with the addition of all essential amino acids (20μg/ml each of adenine sulfate, L-arginine-HCl, L-histidine-HCl, L-menthionine and uracil; 30μg/ml each of L-isoleucine and L-tyrosine; 50μg/ml of L-phenylalanine; 100μg/ml of L-leucine and 150μg/ml of L-valine) except for L-lysine and L-tryptophan which acted as selective factors. This culture was then be poured into 100ml of the same medium and grown overnight or until the $O.D._{600}$ absorbence was in the 0.5-1.0 range, indicating exponential growth.

The yeast was then harvested by centrifugation for 5 min. at 1,400 x g and spheroblasts were produced, as we routinely do in our laboratory, by following the protocol described by Lee (1987) until 50% of all the cells were shown to be osmotically sensitive. Seventy percent confluent MCF-7 cells were then harvested by trypsinization and washed three times in fresh medium (Dulbecco's modified Eagle's medium, DMEM). An aliquot of $5x10^7$ MCF-7 cells in DMEM was layered on top of $5x10^8$ protoplasted yeast cells suspended in a solution of 1M Sorbitol, 10nM $CaCl_2$, 75mM HEPES, pH 7.2. After 60 s at room temperature, 20 ml of DMEM was added slowly and the cells were pelleted by centrifugation at 1,000 xg, resuspended in DMEM with 10% fetal bovine serum and plated in ten T75 tissue culture flasks. Forty-eight hours after the spheroblast fusion, the recipient cells were exposed to drug selection by Geneticin (GIBCO BRL, Grand Island, NY), starting at a concentration of 200μg/ml of media and increased the dose by 100μg/ml each week for the following two weeks.

Tumorigenicity experiments: Since clones of the MCF-7 could not be expanded, in vivo tumorigenicity assays were not performed in this cell line. Tumorigenicity studies were performed with the prostate cell line PC 3 cells as described previously (Zenklusen and Conti, 1996).

BAC selection: In order to construct a single fully contiguous series (contig) of bacterial artificial chromosomes (BACs), we utilized the yeast artificial chromosome (YAC) based physical map of chromosome 7 previously reported by Bouffard, et al. (1997). The entire region of interest, with boundaries at D7S522 and D7S677, is contained in a single YAC contig. Using the sequence-tagged sites (STS) contained in the YAC contig, a series of BACs were isolated and a minimal tiling path was selected spanning the whole region. In all, nine BACs (RG030h15, RG253b13,RG054j07, RG300c03, RG114a06, RG099b05, RG343p13, GS234b20 and RG068p20) were selected spanning about 1.2 Mb.

BAC retrofitting: The selected BACs were retrofitted to express a mammalian selection marker (*neo*) using the Cre-Lox system according to the protocol by Kim, et al. (1998). Briefly, 500ng of freshly prepared BAC DNA were mixed with 50ng of the retrofitting vector pRETRObac and 1 unit of Cre recombinase (Novagen, Madison, WI) in a total volume of 30µl of 1x reaction buffer. After incubation for 60 min. at 37°C, 5 min.at 70°C, 10 min. at RT and 60 min. at 4°C, the total reaction was drop-dialyzed for 3hrs against water on top of Millipore V membranes (13mm, 0.025µm pore, #VSWP 01300) floated in a 24 well plates. The whole reaction was then mixed with 20µl of GeneHogs electrocompetent cells (Research Genetics, Huntsville, AL) and transferred to a chilled 0.1cm electroporation cuvette. The cells were pulsed at 1.8 kV, 200 ohms, 25µF using BioRad Gene Pulser (Hercules, CA). One milliliter of SOC media was then added and the cells were incubated for 60 min. at 37°C, after which aliquots of 200 and 400µl were plated in LB plates containing 50µg/ml of chloramphenicol and kanamycin, prespread with 50µl of 2% X-gal.

The colonies obtained were analyzed for the integrity of the insert by pulse field gel electrophoresis, and correct integration of the retrofitting vector was assessed by PCR as described by Kim, et al. (1998).

B. Results

The initial aim of the experiments was to provide functional evidence of the existence of a tumor suppressor gene in chromosome 7 using microcell fusion experiments. The donor cell line is an intact human chromosome 7 on a murine A9 cell line, obtained from Oshimura and Barret as described previously (Zenklusen, et al. 1994a). The receptor cell line was MCF-7 (Brooks, et al. 1973) as originally proposed in the grant.

Several neo-resistant colonies were obtained in two separate experiments. These colonies grow extremely slowly and several of them become senescent. After several weeks some colonies reached a size that allowed their trypsinization and expansion. Several colonies were eventually expanded and subcultured. However, most of these lines

grew extremely slowly and after several month have not reached confluence. Only 3 lines reached confluence and were frozen. Given the characteristics of the in vitro growth of these cells we decided not to test them for tumorigenicity in vivo because it will take several months to obtain an adequate number of cells and we are concerned about possible in vitro selection during this time.

In view of these results, we decided to screen a variety of other human breast cancer cells to select one (or two) that has the region deleted in order to repeat the microcell and spheroblast fusion experiments in a genetically relevant background. For this purpose we tested 15 microsatellite markers covering all the region of interest in chromosome 7. Since these cell lines have been established for a long time and DNA from the patients is not available we used polymorphic microsatellites with a high frequency of heterozygocity in the general population (usually higher than 0.7). Thus, the presence of homozygocity in several contiguous markers is an indication of possible LOH in the region and the likelihood of homozygosis vs actual LOH can be estimated statistically. We tested six well characterized cell lines derived from breast cancer: MCF-7; MDA MB231; BT 549; Zr 76 1; T 47D, MDA MB 468. Results obtained in this experiments are presented in Table 1. It can be observed that MCF-7 cell line do not present consistent homozygocity at the locus defined by microsatellites D7S496-D7S490. This is the region that we have previously observed to be frequently deleted in breast cancer (Zenklusen, et al. 1994). However, this cell line presents homozygocity in three contiguous markers which are more proximal to the region of interest. The probability of this being a random phenomenon is < 0.005, therefore it is likely that actual LOH was present in the breast cancer that originated this tumor or that allelic losses happens during in vitro adaptation. Although this is an interesting phenomenon which may be related to senescense genes defined by Oshimura and Barret, this problem is not in the scope of this grant and therefore was not followed. It may be studied independently if appropriate funding is obtained.

Two cell lines were found (MDA MB231, an Adenocarcinoma-derived line, and ZR-75-1, a Ductal Carcinoma In Situ-derived line) that contain a large area of homozygocity in the critical markers. Both lines have a similar pattern of homozygocity with 8 consecutive markers presenting the same allelic constitution. The probability that the patients were homozygous in this region is 0.000059; therefore we considered that these cell lines present LOH in the critical region and can be utilized for future experiments as described originally for MCF-7 cells.

We have carried out experiments to inhibit the tumorigenicity of the MCF-7 cell lines by introduction of the retrofitted YACs by means of spheroblast fusions. After some minor setbacks in the experiments due to the sensitivity of the acceptor cells to the polyethyleneglycol (PEG), which were overcome by a slight modification of the published protocol, we produced some stable colonies of MCF-7/YAC hybrids. No significant in vitro phenotype change was observed in the resulting hybrids. However, at this stage we already had the genotype of the MCF-7 cell line due to the unusual senescent phenotype displayed by the hchr7 hybrids and had discovered that this particular cell line didn't present any signs of deletion in the region of the putative tumor

suppressor. Thus a decision was made to start these experiments over again using a relevant cell line. However, as discussed above it was also decided that it will be advantageous in the long term to use BACs instead of YAC's for these experiments.

A group of nine Bacterial Artificial Chromosomes (BACs), with sizes ranging 120-185 Kb, spanning the entire critical region (1.2 Mb) have been selected and retrofitted with a *neo* marker. These smaller DNA clones will also be used with the newly selected cell lines in transfection experiments to determine a more precise location of the functional gene. Additionally, these BACs are being sequenced by Dr. E. Green's laboratory (87% already completed), and the genomic sequence is being used to predict candidate genes that will be screened for mutations in the patient's DNA samples. As discussed above the use of BACs for the functional experiments is advantageous because they have a very low rate of chimerism compared to YACs, the transfection experiments are more straightforward than the fusions, and the stability of the cloned DNA in these vectors is higher.

CONCLUSIONS

1. Microcellfusion experiments using breast cancer cell line MCF-7 gave unexpected results. Unlike previous experiments from our laboratory using murine SCC cell lines and prostate carcinoma MC3 cell line, the introduction of chromosome 7 produces a senescence or impaired in vitro growth.

2. To further investigate this problem we studied the likelihood of LOH in MCF-7 cell and found out that it is unlikely that MCF-7 has LOH in the critical region. However, they present a region of potential LOH proximal to the region of interest which may be related to the phenomenon of senescene. This has not been further investigated because it is outside of the scope of this grant.

3. In order to continue the proposed experiments we screened 5 other breast cancer cell lines and identify two that have a pattern of putative LOH similar to the one in breast cancer patients. These cell lines will be used in the future experiments.

4. Although experiments to transfer large fragments of DNA into the breast cancer cell lines were apparently successful we did not continue the characterization of the hybrids because as described above MCF-7 did not appear to be a relevant recipient cells.

5. New fusion experiments are in progress using the relevant cell lines. However, instead of using YACs for this new experiments we will use BACs as a the DNA donor. Nine BACs have been identified and characterized. They were also retrofitted to include selectable markers and will be used during the second year of the grant.

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Table 1: Allelic pattern of chromosome 7 in breast cancer cell lines.

Marker	MCF7	MDA MB 231	BT 549	Zr-76-1	T47D	MDA MB 468	HS578T	HET
D7S518	L	H	H	H	H	H	H	0.87
D7S515	L	H	H	H	H	H	H	0.82
D7S496	L	H	H	H	H	H	H	0.75
D7S523	H	H	H	H	H	H	H	0.8
D7S486	H	H	H	H	H	H	H	0.81
D7S522	H	L	H	L	H	L	L	0.67
D7S677	L	L	L	L	H	H	H	0.63
D7S633	H	L	L	L	H	H	H	0.62
D7S655	L	L	H	L	L	H	H	0.45
D7S480	H	L	H	L	L	H	H	0.86
D7S490	L	L	L	L	H	H	H	0.79
D7S487	L	L	H	L	H	H	L	0.73
D7S514	H	L	H	L	H	H	L	0.71
D7S500	H	H	H	H	H	H	H	0.87
D7S495	L	H	H	H	H	H	H	0.81
Likelihood		0.000059		0.000059				